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RESEARCH

Phytochemicals, antimicrobial, and sporicidal activities of moss, *Dicranum polysetum* Sw., against certain honey bee bacterial pathogens

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Abstract

Beekeeping is an important agricultural and commercial activity globally practiced. Honey bee is attacked by certain infectious pathogens. Most important brood diseases are bacterial including American Foulbrood (AFB), caused by *Paenibacillus larvae (P. larvae)*, and European Foulbrood (EFB) by *Melissococcus plutonius (M. plutonius)* in addition of secondary invaders, e.g. *Paenibacillus alvei* (*P. alvei*) and *Paenibacillus dendritiformis* (*P. dendritiformis*). These bacteria cause the death of larvae in honey bee colonies. In this work, antibacterial activities of extracts, fractions, and isolated certain compounds (nominated **1–3**) all originated from moss, *Dicranum polysetum* Sw. (*D. polysetum*), were tested against some honey bee bacterial pathogens. Minimum inhibitory concentration, minimum bactericidal concentration, and sporicidal values of methanol extract, ethyl acetate, and *n*-hexane fractions ranged between 10.4 and 18.98, 83.4-303.75 & 5.86–18.98 µg/mL against *P. larvae*, respectively. Antimicrobial activities of the ethyl acetate sub-fractions (fraction) and the isolated compounds **(1–3)** were tested against AFB- and EFB-causing bacteria. Bio-guided chromatographic separation of ethyl acetate fraction, a crude methanolic extract obtained from aerial parts of *D. polysetum* resulted in three natural compounds: a novel one, i.e. glycer-2-yl hexadeca-4-yne-7*Z*,10*Z*,13*Z*-trienoate (**1**, dicrapolysetoate; given as trivial name), in addition to two known triterpenoids poriferasterol (**2**), and γ-taraxasterol (**3**). Minimum inhibitory concentration ranges were 1.4-60.75, 8.12-65.0, 2.09–33.44 & 1.8-28.75 µg/mL for sub-fractions, compounds **1, 2**, and **3**, respectively.

Keywords *Dicranum polysetum* · Antimicrobial activity · *Paenibacillus larvae* · European Foulbrood · Honey bee larvae

Introduction

Finding new bioactive substances of natural sources is a continuous research for therapeutic uses. About 30% of medicines sold worldwide based on natural products (Grabley and Thiericke 1999). In Turkey, the Eastern Black Sea is one of the most important regions for the common medicinal plant diversity and endemic plant richness due to different geographical conditions, high-elevation plateaus, and climate (Kaya and Raynal 2001). The prevalence of medicinal plants has a significant potential for both beekeeping applications and natural product/products development.

The American Foulbrood (AFB) agent, *Paenibacillus larvae (P. larvae)*, and the European Foulbrood (EFB) one, *Melissococcus plutonius (M. plutonius)*, are the main pathogens of honey bee larvae (Evans and Schwarz 2011). Both

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bacterial pathogens lead to larval death and hive collapse, and the beekeeper suffers great economic losses (Genersch 2010). Ingestion of at least ten *P. larvae* spores by larvae with contaminated food is sufficient to develop AFB disease (Hitchcock et al. 1979; Brødsgaard et al. 1998). *P. larvae* only infects unsealed young honey bee larvae, then larval death occurs depending on the ERIC I-V genotype, within 3–12 days, from the beginning of the infection (Hornitzky and Wilson 1989; Ellis and Munn 2005; Beims et al. 2020).

In EFB infection, *M. plutonius* is ingested with contaminated food and multiply rapidly with asymptomatic colonization in the midgut of larvae. Some other bacteria are frequently associated with EFB, including *P. dendritiformis, P. alvei, Enterococcus faecalis*, *Bacillus pumilus*, *Achromobacter eurydice*, and *Brevibacillus laterosporus* as secondary invaders agents (Forsgren et al. 2013; Gaggia et al. 2015; Erler et al. 2018). *P. dendritiformis* is a facultative anaerobic bacterium with rod-shaped, Gram-positive, motile, and round, including cylindrical or oval spores formed in swelling sporangia. Moreover, *P. alvei* is a saprophytic, aerobic bacterium that can usually be isolated from diseased larvae obtained from honey bee colonies infected with *M. plutonius* (Forsgren 2010; Djukic et al. 2012).

The illegal global use of antibiotics by beekeepers against these diseases triggers antibiotic resistance in these bacteria and resistance genes are spreading into the environment (Genersch 2010; Evans 2003). The use of antibiotics in the treatment of AFB is prohibited in European Union countries due to antibiotic residues detected in honey and the spread of antibiotic resistance (Genersch 2010; Forsgren et al. 2018). Oxytetracycline hydrochloride (OTC) is a bacteriostatic antibiotic used in many countries (USA, Canada, and Australia) that inhibits the growth of *M. plutonius* (Thompson and Brown 2001; Richards et al. 2021; Masood et al. 2022). OTC resistance of *P. larvae* increased as a function of the antibiotic concentration applied (Miyagi et al. 2000). Antibiotics are not a sustainable strategy as they only masks the clinical symptoms and signs of AFB and they are ineffective against the bacterial spores that cause the disease (Oldroyd et al. 1989; Stephan et al. 2019). The demands for alternative natural products (e.g. essential oils, plant extracts, and bee propolis) that can prevent and control AFB are increasing (Alonso-Salces et al. 2017). Extracts of many plants are being investigated for their antibacterial and biochemical effects on bees and larvae infected with *P. larvae* (Basile et al. 1998; Akmaz 2001; Chaimanee et al. 2017; Giménez-Martínez et al. 2019; Alpay Karaoğlu et al. 2022).

Dicranum polysetum Sw. (*D. polysetum*) is a moss belonging to the genus *Dicranum*, family *Dicranaceae*, and the division Bryophyta, a group of non-vascular plant species distributed worldwide (Hedenäs and Bisang 2004; Záveská Drábková et al. 2015). Fourteen species of *Dicranum* have been registered from Turkey (Ros et al. 2013; Erata and Batan 2020). Natural compounds, e.g. organic acids and phenolics isolated from some bryophyte species, i.e. of *Sphagnum*, *Dicranum*, *Polytrichum*, *Atrichum*, and *Mnium* genera, show antibiotic properties and are effective on certain bacteria (Nikolajeva et al. 2012), and reported to have high biological activity (Krzaczkowski et al. 2009; Üçüncü et al. 2010; Cheng et al. 2012). Therefore, extracts and fractions of mosses could be an important source as new effective pharmaceutical agents (Wang et al. 2005; Asakawa 2007). However, the potential of the crude extract of *D. polysetum* and the compounds fractionated from the extract for use against bee diseases has not yet been investigated.

The aim of the present study was to evaluate the effects of *D. polysetum* extract, fractions, and relevant certain purified compounds obtained by different solvents against the pathogens of AFB and EFB disease in honey bee larvae. In addition, certain novel secondary metabolites were isolated from *D. polysetum* fractions by means of spectroscopic methods (i.e. NMR, UV, FT-IR, and LC-QTOF-MS).

Materials and methods

General procedures

NMR spectra were recorded on a Bruker spectrometer in $CDCl_3$ using tetramethylsilane as an internal standard at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR spectra. Chemical shifts were expressed in δ (ppm) and coupling constants (*J*) were reported in hertz (Hz). The assistance of ACD NMR programs was also used for the elucidation of isolated compounds. LC-QTOF-MS was obtained on an Agilent 6230 A instrument. UV spectra were obtained with a Spectrostar nano BMG labtech spectrometer. Infrared spectra were obtained with a PerkinElmer 1600 FT-IR (ATR) ($4000-400\ cm^{-1}$) spectrometer. Melting points were determined using Thermovar apparatus fitted with a microscope and are uncorrected. Column chromatography was performed with silica gel 60 (320–400 mesh). Thin-layer chromatography was performed with TLC Silica gel 60 F_{254} aluminum TLC plates, visualized by UV or spraying with vanillin/H_2SO_4 solution followed by warming (Erik et al. 2021a, b).

Moss material

D. polysetum samples were collected from Sis Mountain, 40º52′28.89″ N, 39º06′53.32″ E, altitude 1931 m on Oct^{26th}, 2018 at Trabzon province, Turkey. After laboratory identification, the moss samples were cleaned and dried under appropriate conditions (Smith 1980; Hedenäs and Bisang

2004; Ros et al. 2013; Özdemir and Batan 2017; Erata and Batan 2020). The plant material was identified by one of the co-authors (Prof. Dr. Nevzat Batan) following the protocol of Hedenäs and Bisang (2004). A voucher specimens (Voucher number: KTUB 1613) were stored in the bryophyte collections of the Biology Department, Faculty of Science, Karadeniz Technical University, Turkey.

Extraction and isolation

Dried powdered sample of *D. polysetum* (~1500 g) was macerated with methanol (5 L) three times for 72 h at room temperature (24 ± 1 °C) on stirring. Total extract was evaporated under vacuum at a temperature not exceeding 40 °C using a chiller (-10 °C) to yield crude extract (34.20 g). The crude methanol extract (30 g) was dissolved in MeOH-H_2O (2:8 v/v, 100 mL) and then successively fractionated with *n*-hexane (500 mL x 3), ethyl acetate (500 mL x 3), and water (250 mL) to yield 7.56, 9.57, and 13.25 g fractions, respectively (Narayan et al. 2010). Crude ethyl acetate fraction was used for further phytochemical tests. This fraction (9.57 g) was subjected to CC (Kieselgel 60, 230–400 mesh) using increased polarity of *n*-hexane (100 mL), *n*-hexane:chloroform (1:1, 100 mL), chloroform (100 mL), chloroform-ethyl acetate (1:1, 100 mL), ethyl acetate (100 mL), ethyl acetate-methanol (1:1, 100 mL), and methanol (100 mL) mixture to get six sub-fractions (~120 mL, each, DPE1-6, respectively). After the TLC analysis, the DPE-2 fraction afforded compound **1** (353 mg) given a trivial name dicrapolysetoate.

The DPE-3 fraction (2.49 g) was subjected to CC (Kieselgel 60, 320–400 mesh) using increased polarity of *n*-hexane-chloroform (100:0, 80:20, 60:40, 20:80, and 0:100 mL each), chloroform-ethyl acetate (100:0, 80:20, 60:40, 20:80, and 0:100 mL, each), and ethyl acetate-methanol (100:0, 80:20, 60:40, 20:80, and 0:100 mL each) mobile phase to get 26 fractions (~50 mL each). After TLC control, DPE-3 Fr 5 afforded compound **2** (23.8 mg). After TLC analysis, DPE-4 and DPE-5 fractions were combined and subjected to CC (Kieselgel 60, 320–400 mesh) using increased polarity of *n*-hexane-chloroform (100:0, 95:5, 90:10, 85:5, 80:20, 75:25, 70:30,65:35, 60:40, 50:50, 40:60, 20:80, and 0:100 mL, each), chloroform-ethyl acetate (100:0, 90:10, 80:20,70:30, 60:40, 50:50,40:60, 30:70, 20:80, and 0:100 mL each), and ethyl acetate-methanol (100:0, 80:20, 60:40, 20:80, and 0:100 mL, each) mobile phase to get 41 fractions (~40 mL each). After TLC control, Fr 8–15 (0.71 g) were combined and again was further purified with CC (Kieselgel 60, 320–400 mesh) with increasing polarity of *n*-hexane-ethyl acetate (100:0, 98:2, 96:4,92:8,90:10, 88:12, 86:14, 82:18, 80:20, 75:25, 70:30, 65:35, 60:40, 50:50, 40:60, 30:70, 20:80,10:90, and 0:100 mL, each) to get 30 sub-fractions (SFr) (~30 mL, each). After TLC control, SFr 18 afforded compound **3** (6.2 mg).

The most active ethyl acetate fractions of *D. polysetum* were separated into six sub-fractions with column chromatography using different solvents of increasing polarity. These six obtained sub-fractions were kept in the refrigerator until it was time to test them for antimicrobial activity. These sub-fractions (1–6) were weighed in specific amounts and tested for activity against honey bee pathogens.

Glycer-2-yl hexadeca-4-yne-7Z,10Z,13Z-trienoate (1) Colorless oily; R_f: 0.82 (*n*-hexane-AcOEt, 9.5:0.5); UV (Methanol) λ_{max} (nm): 216, 245, 280. FT-IR (ATR, cm^{-1}): FT-IR (ATR, cm^{-1}): 3405 (-OH), 2934, 2860 (-CH), 2148 (C≡C), 1705 (C=O), 1450, 1373 (C=C) 1268, 1141, 1055, 1033 (C-O); $C_{19}H_{28}O_4$, LC-QTOF-MS: m/z (%) $[M-H]^+$ 319.1960 (15), calc 319.1910. NMR data of new compound **1** are in Table 1.

Poriferasterol (2) R_f: 0.70 (Chloroform-EtOAc, 7:3); NMR data of known compound **2** are in Table 2 (Matin Yekta and Alavi 2008; Yekta et al. 2018).

γ-Taraxasterol (3) R_f: 0.68 (Chloroform-EtOAc, 7:3); NMR data of known compound **3** are in Table 2 (Matin Yekta and Alavi 2008; Yekta et al. 2018).

Antimicrobial activities

Bacteria and cultures

Bacterial strains causing AFB and EFB disease were isolated from honey bees (*Apis mellifera anatoliaca*); workers, larvae, and honey. These samples were characterized by conventional methods (de Graaf et al. 2013). Bacterial DNA samples were amplified by PCR (Bio-Rad Model T100, CA, USA) using universal specific primers (27 F and 1492R) targeting the 16 S rRNA genes. The PCR conditions were adapted according to the study of Demirci et al. (2013). After the nucleotide sequencing of the PCR products, closely related strains were searched from the NCBI GenBank databases (Baş and Karaoğlu 2015; Pınarbaş and Karaoğlu 2017). The NCBI GenBank accession numbers of the identified bacteria strains were listed in Table 3. In addition, the *P. larvae* genotype reference strains *P. larvae* ERIC-I (ATCC 9545), ERIC-II (DSM 25,430), and ERIC-III (LMG 16,252) used in the study were obtained from Samsun Veterinary Control Institute. *P. alvei* (Clone M6) and *M. plutonius* (clone I1) were obtained from Sophia Antipolis, Honeybee Pathology Laboratory Unit (France).

Table 1 ^{1}H and ^{13}C-NMR data of **1** (400/100 MHz, $CDCl_3$, δ, ppm)

No	^{1}H	^{13}C	APT
1	-	172.8	C
2	2.12 (d, H-2, 2 H)	33.4	CH_2
3	2.34 (brs, H-3, 2 H)	17.0	CH_2
4	-	78.5	C
5	-	79.2	C
6	2.92 (m, H-6, 2 H)	18.4	CH_2
7	5.36 (m, 1 H)	128.7	CH
8	5.36 (m, 1 H)	129.1	CH
9	2.82 (m, H-9, 2 H)	28.0	CH_2
10	5.36 (m, 1 H)	127.3	CH
11	5.36 (m, 1 H)	126.9	CH
12	2.82 (m, H-12, 2 H)	25.4	CH_2
13	5.36 (m, 1 H)	126.3	CH
14	5.36 (m, 1 H)	131.9	CH
15	1.51 (m, H-15, 2 H)	23.9	CH_2
16	0.97 (brs, H-16, 3 H)	14.3	CH_3
1’	5.36 (m,1 H)	68.9	CH
2’, 1”	4.23 (m, 4 H)	62.1	CH_2

Table 2 ^{1}H and ^{13}C-NMR data of **2** and **3** (400/100 MHz, $CDCl_3$, δ, ppm, J = Hz)

C/H	2		3	
	^{1}H	^{13}C (APT)	^{1}H	^{13}C (APT)
1		37.2		38.8
2		31.7		27.1
3	3.52, m	71.8	3.21, m	79.0
4		42.3		38.8
5		140.7		55.2
6	5.35, brd	121.7		18.3
7		31.9		33.9
8		31.9		41.9
9		50.1		51.0
10		36.5		37.0
11		21.1		21.2
12		39.7		27.6
13		42.3		29.0
14		56.9		42.7
15		24.3		27.1
16		28.9		36.7
17		56.0		34.6
18	0.68, s, 3 H	11.9		48.0
19	1.01, s, 3 H	19.2		36.8
20		40.6		137.8
21	1.02, d, 3 H	21.2	5.26, brd	125.8
22	5.16	138.3		42.7
23	5.02	129.2	0.82, s, 3 H	28.1
24		51.2	0.88, s, 3 H	15.4
25		31.9	0.89, s, 3 H	16.9
26	0.92, d, 3 H	21.2	1.08, s, 3 H	15.6
27	0.79, d, 3 H	19.0	0.92, s, 3 H	14.1
28		25.4	0.78, s, 3 H	17.0
29	0.82, t, 7.6, 3 H	12.3	0.98, d, 6.6, 3 H	22.7
30			1.63, brs, 3 H	20.9

MYPGP (Mueller Hinton Broth (1%), yeast extract (1.5%), K_2HPO_4 (0.3%), Glucose (0.2%), Sodium pyruvate (0.1%), and agar (15%) (Merck, Germany) medium was used for the culture of all bacteria (Dingman and Stahly 1983; Sevim et al. 2021). Cultures were inoculated on MYPGP agar and incubated with a 5% CO_2 incubator at 36 ± 1 °C for three days under microaerophilic conditions (Alpay Karaoğlu et al. 2022).

Antimicrobial activities of *D. polysetum* extract, fractions, and isolates

Potential antimicrobial activities of *D. polysetum* extracts and fractions were tested against honey bee tested pathogens using the agar well diffusion method (Perez et al. 1990; CLSI 2015). The bacterial culture was grown in a shaking incubator overnight to produce a bacterial turbidity of 1×10^8 CFU/mL in MYPGP broth. McFarland 0.5 bacterial turbidity was prepared and spread with a sterile cotton swab over the surface of MYPGP agar in 90 mm diameter petri-dishes. Wells of 5 mm diameter at 3 cm intervals were made in MYPGP agar petri-dishes using sterile glass tubes. A 50 µL of moss extract and fractions were dropped into each well. The inhibition zone diameters were measured with a ruler after the petri-dishes were incubated for three days at 36 ± 1 °C with a 5% CO_2 incubator (Nordström and Fries 1995; Sevim et al. 2021). Tetracycline (CT0054B, Oxoid, England) (30 µg) was used as a standard drug control (Mejias 2020) as well as solvents.

Minimum inhibition (MIC) and minimum bactericidal concentrations (MBC)

MIC and MBC tests were performed for the moss extract, fractions, and compounds (**1–3)** that could prevent the development of AFB and EFB agents. Bacteriostatic and Bactericidal concentrations (µg/mL) were determined by applying the micro-dilution method in ELISA (96 well) plates (CLSI 2015). Bacterial turbidity was prepared as 0.5 McFarland or 1×10^8 CFU/mL. The 10 µL bacterial solution was added to each well and incubated for 48 h for *P. alvei* and *P. dendritiformis*, and 72 h for *P. larvae* and *M. plutonius* at 36 ± 1 °C with a 5% CO_2 incubator (Arai et al. 2012). The MIC value was taken from the no-growth diluted well, i.e. the lowest antimicrobial agent concentration. For MBC, 10 µL dilutions were taken from non-growing wells and were dropped onto MYPGP agar plates. The concentration of the substance in the well with no growth was determined as the MBC value (Table 4).

Table 3 Origin, characteristics of AFB and EFB causative strains used in the study, and antimicrobial susceptibility to *D. polysetum* crude methanol extract

Strains No	Accession No	Location	Origin	Ant. Res.	Similarity Strains	GenBank No	Sim(%)	AMP(mm)	IZ (mm)	MIC(µg/mL)	MBC(µg/mL)
PB3.2 A	KU598689	Rize	HB	-	*P. l.* Ymb1 *P. l. l.*	EF187246.1 AY030079.1	99 99	35 ± 0.3	18 ± 0.1	0.36 ± 0.0	2.92 ± 0.1
PB3.2B	KU598690	Rize	HB	-	*P. l.* 03-189 *P. l. l.*	DQ079623.1 AY030079.1	99 99	35 ± 0.3	15 ± 0.1	0.73 ± 0.0	2.92 ± 0.1
PB3.3 A	KU598692	Rize	HB	-	*P. l.* 02-130 *P. l. l.*	DQ079622.1 AY030079.1	100 100	35 ± 0.3	20 ± 0.1	0.36 ± 0.0	0.72 ± 0.0
PB4A	KU598693	Samsun	L	-	*P. l.* 02-130 *P. l. l.*	DQ079622.1 AY030079.1	100 100	50 ± 0.5	15 ± 0.1	0.73 ± 0.0	2.92 ± 0.1
PB5A	KU598694	Samsun	L	-	*P. l.* Ymb1 *P. l. l.*	EF187246.1 AY030079.1	100 100	40 ± 0.4	28 ± 0.2	0.36 ± 0.0	0.73 ± 0.0
PB6A	KU598696	Samsun	HB	-	*P.l.* Ymb1 *P. l. l.*	EF187246.1 AY030079.1	99 99	50 ± 0.5	34 ± 0.3	0.36 ± 0.0	0.73 ± 0.0
PB31B	MZ673470	Samsun	L	E, TY, TE	*P. l.* 03-525 *P. l. l.* PL35	DQ079620.1KT363742.1	97 97	15 ± 0.1	18 ± 0.1	6.91 ± 0.1	13.83 ± 0.1
PB35	MW227606	Bayburt	HB	E	*P. l. l.* B-3553 *P. l.* 03-189	KT363740.1 DQ079623.1	99 99	10 ± 0.1	15 ± 0.1	27.65 ± 0.2	55.28 ± 0.3
SV27B	MZ156075	Samsun	L	E, TY, TE, T	*P. l.* 02-130 *P. l. l.* B-3555	DQ079622.1 KT363739.1	99 99	10 ± 0.1	18 ± 0.1	6.91 ± 0.1	13.83 ± 0.1
PB33	MZ673473	Rize	H	E, TY, T	*P. alvei* Y2 *P. alvei* RMS01	KX266960.1 JX437031.1	99 99	10 ± 0.1	15 ± 0.1	13.83 ± 0.1	27.65 ± 0.2
PB22F	MZ156073	Rize	H	E, T	*P. d.* PP *P. d.* KP	KX082752.1 KX083535.1	99 99	36 ± 0.3	18 ± 0.1	13.83 ± 0.1	27.65 ± 0.2
PB31A	Lab stock	Samsun	L	E	*P. d.* ANSK05 *P.* sp. BAB-3421	KT152690.1 KF917151.1	99 99	-	18 ± 0.1	13.83 ± 0.1	27.65 ± 0.
ERIC-I	DSMZ7030	-	Ref.	-				Nt	18 ± 0.1	6.91 ± 0.1	13.83 ± 0.1
ERIC-II	DSMZ25430	-	Ref.	-				Nt	15 ± 0.1	27.65 ± 0.2	55.28 ± 0.5
ERIC-III	LMG16252	-	Ref.	-				Nt	16 ± 0.1	13.83 ± 0.1	27.65 ± 0.2
P. alvei M6	Lab stock*	-	-	-				Nt	15 ± 0.1	27.66 ± 0.2	55.28 ± 0.5
M. p. I1	Lab stock*	-	-	-				Nt	20 ± 0.2	6.91 ± 0.1	13.83 ± 0.1

H: Honey, HB: Worker bee, L: Larvae, *P.l.: P. larvae, P.l.l.: P. larvae* subsp. *larvae*, *P.d: P. dendritiformis, P.* sp.: *Paenibacillus* sp., Ref.; Reference strain,Ant.Res.; Antibiotic resistance, E; Erythromycin, TY; Tylosin, TE; Tetracycline, AMP; Ampicillin, T; Oxytetracycline, (-); no antibiotic resistance, IZ: İnhibition zone. Lab stock*: Sophia Antipolis, Laboratory Unit of Honey-bee Pathology (France), Nt; not tested

Table 4 Antimicrobial activity of *D. polysetum* extract and fractions against *P. larvae* strains

Extract and fractions	Stock conc. (µg/mL)	Effectiveness of extract and fractions against *P. larvae* strains by the agar well diffusion method (mm)						
		PB3.2B	PB13.1B	PB31B	PB31A	PB35	SV27B	SV30B*
Methanol extract	26,700	25 ± 0.2	24 ± 0.2	20 ± 0.2	24 ± 0.2	12 ± 0.1	20 ± 0.2	20 ± 0.2
Water fraction	36,400	-	-	-	-	-	-	-
EtOAc fraction	15,000	20 ± 0.2	20 ± 0.2	17 ± 0.1	20 ± 0.2	11 ± 0.1	18 ± 0.1	17 ± 0.1
n-Hexane fraction	24,300	19 ± 0.1	19 ± 0.1	19 ± 0.1	18 ± 0.1	10 ± 0.1	19 ± 0.1	18 ± 0.1
Tetracycline[a]	30	30 ± 0.6	30 ± 0.6	30 ± 0.6	-	35 ± 0.8	30 ± 0.6	15 ± 0.3
Extract and fractions	Stock conc. (µg/mL)	Sporicidal and bactericidal activity of extract and fractions against *P. larvae* B31B (µg/mL)						
		Vegetative form MIC		Vegetative form MBC		Spore germination inhibition		
Methanol extract	26,700	10.4 ± 0.2		83.4 ± 0.4		10.43 ± 0.1		
EtOAc fraction	15,000	11.72 ± 0.2		93.75 ± 0.4		5.86–11.72 ± 0.1		
n-Hexane fraction	24,300	18.98 ± 0.2		303.75 ± 0.5		18.98 ± 0.1		

*; *P. larvae* SV30B Acsession Number MZ673472, Location: Samsun, (-); No İnhibition zone observed. a; Positive control for antibacterial assay

Sporicidal activity

The effectiveness of moss extracts, i.e. methanol, ethyl acetate, and *n*-hexane fractions in stopping germination of spore form of *P. larvae* was investigated. Spore-producing *P. larvae* PB31B was tested. This strain was incubated on MYPGP agar medium for 5 days and kept at 4 °C for 15–20 days. Spore harvest from the culture was done in cold, sterile distilled water using sterile swabs and placed in a hot water bath (72 ± 1 °C) for 10 min to kill vegetative bacterial cells. Serial dilutions were prepared to determine the number of viable spores per mL, and 100 µL was plated on MYPGP agar. To obtain a concentration of 2.0×10^6 spores/mL, a stock spore suspension was made for bacterial strain (Okayama et al. 1997). The sporicidal activities of *D. polysetum* extract and fractions against the spore form of *P. larvae* PB31B strain were analyzed by the microdilution technique (Table 4).

Results

Antimicrobial activities of *D. polysetum* against certain honey bee pathogens

Mosses could be considered as a source of novel biologically active compounds. Compared to plants, phytochemical and biological activities of mosses in bee diseases have been less studied. Determination of antibiotic susceptibility and resistance characteristics of the strains used is important in evaluating the efficacy of *D. polysetum* extracts, fractions, and individual compounds against AFB and EFB agents. In this study, the potential antimicrobial activities of *D. polysetum* extract, fractions, and isolated compounds (nominated **1**–**3**) (Fig. 1) were investigated. *D. polysetum* was found to have strong antimicrobial activity against *P. larvae*, *P. dendritiformis, P. alvei*, and *M. plutonius*. Antibiotic resistance profile of bacteria causing AFB and EFB are listed in Table 3. Antimicrobial resistance in bacteria has been a growing problem in recent years. The tested moss extract was found to have an 18-mm zone of inhibition against *P. larvae* SV27B and *P. larvae* PB31B, strains resistant to antibiotics (tylosin, tetracycline, and oxytetracycline) used in bee diseases control. Remarkable antimicrobial activities of moss extracts, fractions, and compounds against antibiotic-sensitive and -resistant strains were observed (Table 4).

Noticeably, methanol extract of *D. polysetum* exhibited antimicrobial activities against AFB and EFB agents (Table 3). MIC and MBC values of the extracts against high antibiotic- resistant strains, i.e. PB31B and SV27B were 6.91 & 13.83 µg/mL, respectively, highest values were observed for PB35 and ERIC-II strains. *M. plutonius* was the most sensitive strain (MIC and MBC were 6.91 & 13.83 µg/mL, respectively). Activities against other tested EFB agents were similar to those of the ERIC-II and ERIC-III strains. Obtained results suggest potent activity of the extract regardless of the strains or antibiotic resistance. The elucidation of the mechanism of action of tested extracts or purified novel compounds will be subjected in further studies.

Antimicrobial activities of aqueous, ethyl acetate, and *n*-hexane fractions of the methanolic extract of *D. polysetum* against *P. larvae* was determined (Table 4). Aqueous fraction was not effective, but ethyl acetate and *n*-hexane fractions were strong antimicrobial (11.72 & 18.99 µg/mL, respectively) and sporicidal (5.86–11.72 & 18.98 µg/mL, respectively) agents. PB35 strain was found to be less susceptible (inhibition zones ranged 10–12 mm) than other tested strains (19–25 mm) (Table 4).

The six sub-fractions of the ethyl acetate exhibited potent antimicrobial activities against *P. larvae*

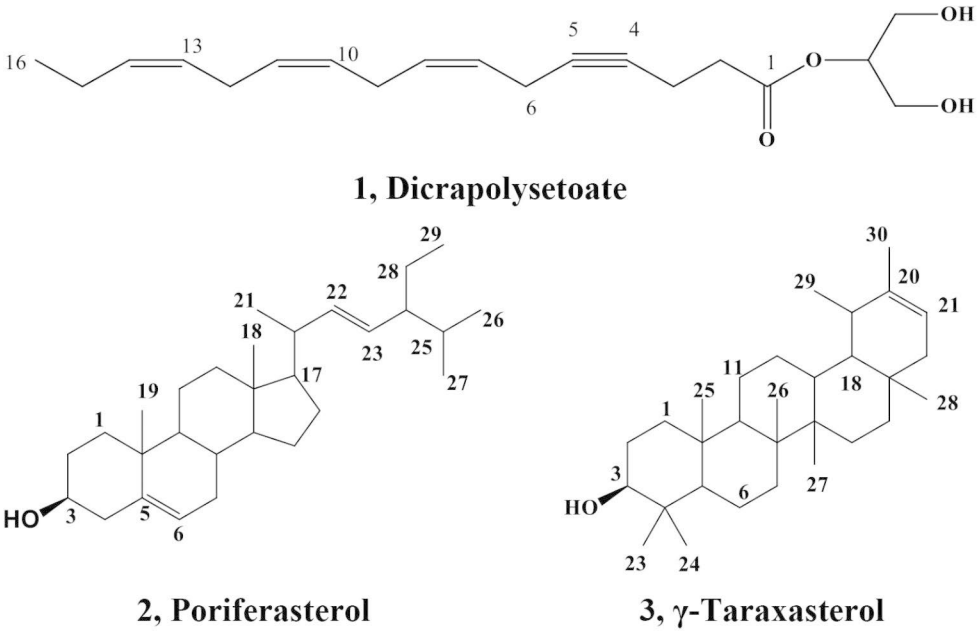

Fig. 1 Chemical structure of isolated compounds (1–3) from *Dicranum polysetum* Sw.

(MIC range 1.4–60.78 µg/mL), while was between 2.8–92.5 µg/mL for other strains (Table 3). All subfractions showed antimicrobial activity values less than 100.0 µg/mL as well as strong anti-*Paenibacillus* effectiveness.

Subfraction 5 gave the highest MIC values, range 1.4–5.6 µg/mL, against all tested bacteria (Table 5) regardless the antibiotic-resistance profiles of the strains.

Subfraction 2 was effective at 7.6 µg/mL in ERIC-II reference strain or *M. plutonius*, while was between 15.25 and 60.78 µg/mL against AFB agents. Compound **1** has high activity against *P. larvae* PB31B (8.12 µg/mL), ERIC-I reference strain (16.25 µg/mL) and *P. dendritiformis* PB31A (16.25 µg/mL), while range was 32.5–130.0 µg/mL against other tested bacteria. Compound **2** showed strong antimicrobial activity against AFB and EFB agents (range 2.09–33.44 µg/mL) (Table 5) as well as PB31B (2.09 µg/mL), resistant to tetracycline and tylosin. MIC (8.36 µg/mL) was recorded for *M. plutonius*, *P. dendritiformis* PB31A, and reference strain (ERIC-I and ERIC-III genotype). Subfraction 3 (DPE-3) was active against AFB and EFB agents (range 4.57–36.56 µg/mL). DPE-4 and DPE-5 fractions were combined, while compound **3** showed strong antimicrobial activity (range 1.8–28.75 µg/mL) against AFB and EFB agents (Table 5), highest antimicrobial activity was observed against *P. larvae* PB31B, *P. larvae* SV27B, and *P. dendritiformis* PB31A. Contrarily, fraction DPE-4 has low antimicrobial activity, while fraction DPE-5 has very high antimicrobial activity (1.4–5.6 µg/mL) against tested microorganisms.

Structure elucidation of 1 isolated from ethyl acetate fraction of *D. polysetum*

The methanol extract of the aerial part of *D. polysetum* was successively fractionated with *n*-hexane, EtOAc, and water. Repeated column chromatography for the most active EtOAc fraction led to the isolation of three compounds (Fig. 1). The structures of the isolated compounds were determined by spectroscopic methods, including NMR, UV, FT-IR, and LC-QTOF-MS. Compound **1** was obtained as oily. The molecular formula was determined as $C_{19}H_{28}O_4$ from the LC-QTOF-MS data (*m/z* 319.1960 $[M-H]^+$, calc 319.1910). The FT-IR (ATR) spectrum of compound **1** gave the presence of a hydroxyl group (3405 cm^{-1}), an acetylenic (2148 cm^{-1}), C = C (1450, 1373 cm^{-1}), and a carbony1 group (1705 cm^{-1}). The UV spectrum of **1** showed absorption maxima at 216, 245, 280 nm typical of a higher unsaturated acetylene-containing fatty acid derivative (Paul and Fenical 1980; Ichikawa 1984). The ^{1} H NMR spectrum of **1** showed six olefinic protons at δ 5.36 ppm (H-7,8, H-10,11, and H-13,14) and ^{13} C NMR spectrum revealed six olefinic CH peaks at δ 126.3-131.9 ppm, two acetylenic carbons at δ 78.5 (C-4) and 79.2 (C-5), six methylene, and one ester carbonyl at δ 172.8 ppm. The presence of the glyceryl moiety was indicated by ^{1} H NMR resonances of one oxymethine proton at δ 5.36 ppm (m, 1 H, H-1'), together with two sp^3 oxymethylene protons at δ 4.23 ppm (m, 4 H, H-2', 1'') and carbon peaks at δ 68.9 (C-1'), and 62.1 (C-2', 1'') which was substituted by ester linkage at the C-1 position of a fatty acid moiety of compound **1**. The NMR data of **1** (Table 1) were very similar to those of the higher unsaturated acetylene-containing fatty acid (16:4, n-3 and 18:4, n-3) derivative but

Table 5 Antimicrobial activity for the ethyl acetate sub-fractions and compounds **1–3** isolated from *D. polysetum* against AFB and EFB agents (MIC values, µg/mL)

EtOAc Sub-frac. /Comp.	Stock Conc(µg/mL)	ERIC I	ERIC II	ERIC III	PB3.2B	PB6A	SV27B	PB31B	PB35	PB22F	PB31A	M6	PB33	*M. p.* I1
DPE-1	28,800	11.25 ± 0.1	45.0 ± 0.1	45.0 ± 0.2	22.5 ± 0.1	45.0 ± 0.2	22.5 ± 0.2	5.62 ± 0.0	11.25 ± 0.1	45.0 ± 0.2	90.0 ± 0.2	45.0 ± 0.2	45.0 ± 0.2	22.5 ± 0.1
DPE-2	77,800	15.20 ± 0.1	7.6 ± 0.0	3.8 ± 0.0	60.78 ± 0.2	15.19 ± 0.1	60.78 ± 0.2	7.6 ± 0.0	30.40 ± 0.1	30.39 ± 0.2	30.39 ± 0.2	30.39 ± 0.2	30.39 ± 0.2	7.6 ± 0.0
DPE-3	23,400	4.57 ± 0.0	18.28 ± 0.1	18.28 ± 0.1	4.57 ± 0.0	9.14 ± 0.0	9.14 ± 0.0	18.28 ± 0.1	9.14 ± 0.0	9.14 ± 0.0	9.14 ± 0.0	36.56 ± 0.2	18.28 ± 0.1	9.14 ± 0.0
DPE-4	144,600	28.24 ± 0.1	14.12 ± 0.1	7.06 ± 0.0	< 14.12 ± 0.1	14.12 ± 0.1	56.48 ± 0.2	14.12 ± 0.1	7.6 ± 0.0	28.24 ± 0.1	14.12 ± 0.1	28.24 ± 0.1	14.12 ± 0.1	7.06 ± 0.0
DPE-5	28,700	2.80 ± 0.1	5.6 ± 0.0	2.8 ± 0.0	< 2.80 ± 0.0	5.6 ± 0.0	< 2.80 ± 0.0	1.4 ± 0.0	2.80 ± 0.0	5.6 ± 0.0	5.6 ± 0.0	5.6 ± 0.0	5.6 ± 0.0	2.8 ± 0.0
DPE-6	78,500	30.66 ± 0.1	30.66 ± 0.2	15.33 ± 0.1	< 7.66 ± 0.0	30.66 ± 0.1	15.33 ± 0.1	7.66 ± 0.0	30.66 ± 0.1	61.33 ± 0.2	61.33 ± 0.2	92.65 ± 0.3	30.66 ± 0.2	15.33 ± 0.1
1	20,800	16.25 ± 0.1	65.0 ± 0.2	32.5 ± 0.1	Nd	Nd	32.0 ± 0.1	8.12 ± 0.0	Nd	130.0 ± 0.3	16.25 ± 0.1	32.5 ± 0.2	65.0 ± 0.2	32.5 ± 0.2
2	10,700	8.36 ± 0.0	16.72 ± 0.1	8.36 ± 0.0	Nd	Nd	16.72 ± 0.1	2.09 ± 0.0	Nd	33.44 ± 0.2	8.36 ± 0.0	33.44 ± 0.2	16.72 ± 0.1	8.36 ± 0.1
3	4600	28.75 ± 0.1	28.75 ± 0.1	14.38 ± 0.1	Nd	Nd	7.2 ± 0.0	1.8 ± 0.0	Nd	28.75 ± 0.1	3.6 ± 0.0	14.38 ± 0.1	14.4 ± 0.1	28.75 ± 0.2
$CHCl_3$	-	-	-	-	-	-	-	-	-	-	-	-	-	-

DPE: *D. polysetum* ethyl acetate sub-fraction, *P. larvae* strains; PB3.2B, PB6A, SV27B, PB31B, PB35, ERIC I; DSMZ 7030, ERIC II; DSMZ 25,430, ERIC III; LMG 16,252, *P. dendritiformis* strains; PB22F, PB31A, *P. alvei strains;* M6, PB33, *Melissococcus plutonius* I1 clone; Mp I1, Nd: Not determined. $CHCl_3$; Chloroform control

differed from known compounds with regard to the glyceryl moiety (Paul and Fenical 1980; Borel et al. 1993; Klavina et al. 2015). In the present work, compound **1** (a novel substance) was elucidated as glycer-2-yl hexadeca-4-yne-7*Z*,10*Z*,13*Z*-trienoate. In addition, structures of two known triterpenoids (poriferasterol, **2**, and *γ*-taraxasterol, **3**) from the ethyl acetate fraction of *D. polysetum* were also isolated.

Discussion

The antimicrobial activities of the ethanol extract of three moss species (*Dicranum polysetum*, *Calliergonella cuspidata*, and *Hypnum cupressiforme*) against various bacteria were different, e.g. Altuner et al. (2014) assumed weak activity. However, in 13 different mosses, including *D. polysetum*, zone of inhibition ranged 9–15 mm against *Bacillus cereus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* (Borel et al. 1993; Gahtori et al. 2011) found that petroleum ether, methanol, and chloroform extracts of *D. undulatum* had effective MIC (range 0.65–2.50 µg/mL) and MBC (range 1.25-5.00 µg/mL) against *M. plutonius* strains. These findings reveal the importance of selecting solvents in extraction process. All the three substances used in our study that purified by crude methanol extract, ethyl acetate, and hexane fractions of *D. polysetum* were antimicrobial against AFB- and EFB-causing bacteria, even at low concentrations.

On the other hand, antibiotic-resistant isolates of *P. larvae* have been reported in the United States, Canada, and Argentina (Miyagi et al. 2000; Evans 2003; Alippi et al. 2007). Antibiotic use in controlling foulbrood is permitted in many countries (Thompson and Brown 2001). Consequently, resistant bacterial strains complicate treatment, prolong duration of infection, and adversely affect other types of native bacteria (Miyagi et al. 2000; Evans 2003).

Antimicrobial activity of tested fractions against antibiotic-sensitive *P. larvae* was effective at low concentration (0.36–0.73 µg/mL), but in antibiotic-resistant *P. larvae*, concentration increased to 6.91–27.65 µg/mL (Tables 4 and 5). *Paenibacillus* spp. are spore-forming bacteria and the MIC value was determined at the same or similar concentrations as the inhibitory value of spore germination. MBC of crude methanol extract at low concentration (range 0.72 to 55.28 µg/mL) is promising to prevent bacterial diseases in honey bee larvae by inhibiting spore germination.

In the agar-well diffusion experiment, compounds **1–3** inhibited the growth of *Paenibacillus larvae, P. alvei*, *P. dendritiformis*, and *M. plutonius*. Use of extracts or molecules, e.g. antimicrobial fatty acid derivatives or triterpenoids is an alternative way in preserving honey bee larvae (Beuchat 1989). Supporting our finding for Compound **1**, a

fatty acid derivative, plant extracts including essential fatty acids were in vitro effective against *P. larvae* (Mărghitaş et al. 2011; Özkırım et al. 2012; Boligon et al. 2013). Conversely, six chemotypes (acetates) of *Thymus vulgaris* didn't inhibit bacterial growth of EFB (Wiese et al. 2018). The effect of alpha-terpineol against *M. plutonius* was undetected, but MIC values; 292.2 ppm for *P. alvei* and 489.5 ppm for *P. dendritiformis* were reported. Poriferasterol and γ-taraxasterol belong to organic compounds known as triterpenoids. γ-Taraxasterol is a pentacyclic-triterpene and is found in a few plants, including chicory, arnica, and dandelion, *Taraxacum officinale*. Pharmacological effects of taraxasterol, e.g. antitumor, anti-inflammatory, antimicrobial (Zhang et al. 2012); antioxidant, hepatoprotective (You et al. 2010; Aggarwal et al. 2016), and anti-allergic (Mabona et al. 2013) activities have been described. The variation in antimicrobial activity between the pure compound and the subfraction may be due to the synergistic effect.

Conclusion

Recently, new strategies in combating diseases are highly requested, particularly with the use of natural products. The present study aimed to investigate potent efficacy of the moss, *Dicranum polysetum*; extracts, fractions, and isolates against antibiotic-sensitive and -resistant bacterial strains infecting honey bee. Bio-guided chromatographic separation yielded three natural compounds, including a novel one, i.e. glycer-2-yl hexadeca-4-yne-7*Z*,10*Z*,13*Z*-trienoate (**1**), and two known triterpenoids poriferasterol **(2)**, and γ-taraxasterol) **(3)**. As far as we know these compounds were isolated and identified for the first time from *D. polysetum.* Crude methanol extract was observed to have significant anti-*P. larvae* activity in the ethyl acetate fractions. The lowest concentration exhibited antimicrobial activity of methanolic extract was 10.4 µg/mL, while the highest one was 18.98 µg/mL for *n*-hexane fraction. This MIC value is a sporicidal dose as well. The crude methanol extract of *D. polysetum*, ethyl acetate/ n-hexane fractions, and EtOAc sub-fractions were found to have potent antimicrobial activity against AFB and EFB-causing bacteria. Ethyl acetate fractions and sub-fractions showed potent antimicrobial activities against tested honey bee larvae pathogens in the concentration range of 1.8–130.0 µg/mL. However, compounds **2** and **3** were the most effective in a range 1.8 to 33.44 µg/mL MIC. In a conclusion, the current study can contribute to the unveiling of novel alternative extracts and compounds that can be used to protect honey bee larvae from infectious bacterial diseases.

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Declarations

Conflict of Interest The authors declare no conflict of interest.

Ethical approval Not applicable, because the research was not on humans or animals.

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